UNIVERSITY OF PENNSYLVANIA

TISSUE CULTURE STUDIES SHOWING THE EFFECT OF DIPHTHERIA TOXIN, TOXOID, AND T-A MIXTURE UPON FIBROBLASTS OF CHICK EMBRYO HEARTS

M. G. H. MASON

A THESIS

IN BACTERIOLOGY

PRESENTED TO THE FACULTY OF THE GRADUATE SCHOOL IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

PHILADELPHIA
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Tissue Culture Studies Showing the Effect of Diphtheria Toxin, Toxoid and T-A Mixture Upon Fibroblasts of Chick Embryo Hearts

The purpose of this study is twofold: First to determine the differential effect of toxin, toxoid and T-A mixture upon the fibro-blasts from explants of 7-day chick embryo hearts, cultivated *in vitro*, according to the Lewis technique; and second to determine whether such cultures might be suitable for standardizing toxins.

In general the method of study was that of Lewis (14) 1920. Seven-day chick embryo hearts which were cut in small pieces, —explants—were used throughout the experiments. Incubation was at 37°C. At the end of 48 hours, the cultures were examined to determine the migration of fibroblasts. Those pieces showing no active fibroblasts were discarded; while those showing fibroblasts were retained for experimental purposes. The cover glasses were removed from the slides and with a fine capillary pipette, the tissues with their fibroblasts were bathed with various dilutions of the test solutions; the controls being made from each embryo used, were bathed with Locke-Lewis solution. It was noted that Carrel (3) 1923, when using plasma as a medium of growth for his tissue cultures, often withdrew the fluid medium with an aspirator or a pipette and introduced new fluid every 2 or 4 days depending upon the type of culture.

The first experiments were carried on with diphtheria toxin 1–400 and 1–300 dilutions, the observations extending over a period of 144 hours. As the greatest changes took place within the 24- to 48-hour period after the toxin was added (i. e. 72 hours to 96 hours from the beginning of the experiments), it was decided to discard the slides after that period of time had elapsed.

Readings were made at the end of 72 hours and at the end of 96 hours, to determine the presence or absence of living fibroblasts. For each dilution of the experimental substance, results were tabulated from at least 100 pieces of chick embryo heart showing fibroblasts (this number did not include the pieces which were used as controls). The results were recorded both numerically and in percentage. The percentage of pieces showing ab-

sence of living fibroblasts at varying time intervals was recorded and graphs made.

Investigators have, for some time, tried the effect of various bacteria and toxins upon small living organisms. In reviewing the literature, as regards the effect of Corynebacterium diphtheriae "by-products" on minute organisms, and in particular their effect upon tissue cultures grown *in vitro*, we find:

Levaditi and Muttermilch (8, 9) 1913, (10) 1914 and Levaditi (11) 1928, experimented with diphtheria toxin and antitoxin on chick embryo hearts cultivated *in vitro*. However their procedure differs from ours in that the medium used for growing these cultures was plasma. Then too, they used embryonal chicks that were 12 to 20 days old.

In our experiments we used Locke-Lewis solution; we also used comparatively young chick embryos (7-day) because of better growth of fibroblasts, according to Lewis and Lewis (13) 1924. Then too, we used a definite number of M. L. D. doses to the cc.

Levaditi and Muttermilch (8) 1913, used diphtheria toxin prepared in the M. Martin Laboratory, but there is no mention made as to the strength of toxin used. In their experiments, pieces of heart were placed in contact with the toxin for a definite period of time, then removed and allowed to grow in plasma. In our experiments tissues were allowed to grow for 48 hours to ascertain if fibroblasts would appear. Thus if there was no growth at that time, the slides were discarded. In that way one factor of variation was eliminated, for even under the most favorable circumstances, we are told by Lewis and Lewis (12) 1911, also Goodrich and Scott (5) 1922, that pieces vary in growth, even when taken from the same organ of the same embryo and apparently treated in the same way. Having made certain as to the presence of the fibroblasts, they were exposed to the action of the varying test solutions for at least 48 hours, as already stated.

In the experiments of Levaditi and Muttermilch, bouillon was used as a control for the toxin; in our experiments Locke-Lewis solution was used as a control,—the same solution that was used originally to make the varying dilutions.

Smyth (19) 1915, used tissue cultures grown in plasma, and according to his technique the following results were obtained:

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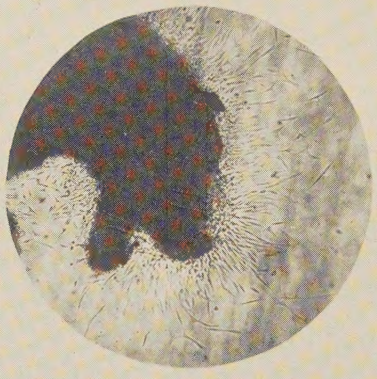


PLATE Ia
Normal Growth of Fibroblasts
48-hour reading

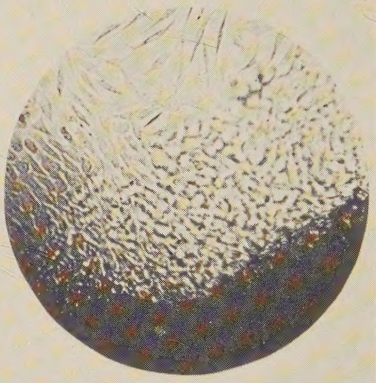


PLATE Ib
Normal Growth of Fibroblasts
48-hour reading

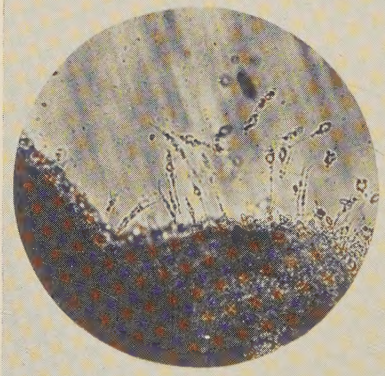


PLATE II
Fibroblasts showing Vacuolated Condition

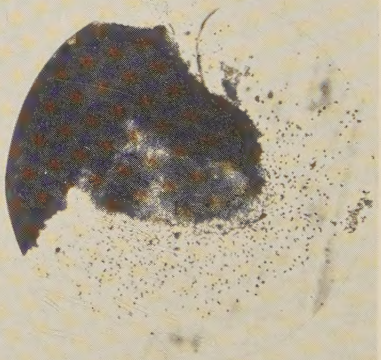


PLATE III
Fibroblasts showing Rounded Up Condition

"Diphtheria toxin has a quantitative inhibiting action on all tissue growth and on heart tissue pulsations, the action being greatest on nervous tissue and least on heart tissue growth. If the dose is not too heavy, tissues recover activity in 24–48 hours, due probably to antitoxin production. Antitoxin prevents or lessens the deleterious action of toxin."

Strangeway (20) 1924, in reference to tissue cultures, states that changes can be produced experimentally by the addition of bacterial toxins to the medium.

Kimura (6) 1930, studied the cellular products of exchange on the fibroblast cultures of chickens.

Takenouchi (21) 1918, found that diphtheria toxin had no effect on paramecia.

Philpott (18) 1928, determined that diphtheria toxin had no appreciable effect on the three species of paramecia he tested.

Tunnicliff (22) 1929, experimented with diphtheria toxin on paramecia and found that the toxic action could only be determined in a crude way because the toxin was not strong enough to be diluted; however, she found that her experimental procedure may be useful in determining the strength of antitoxin.

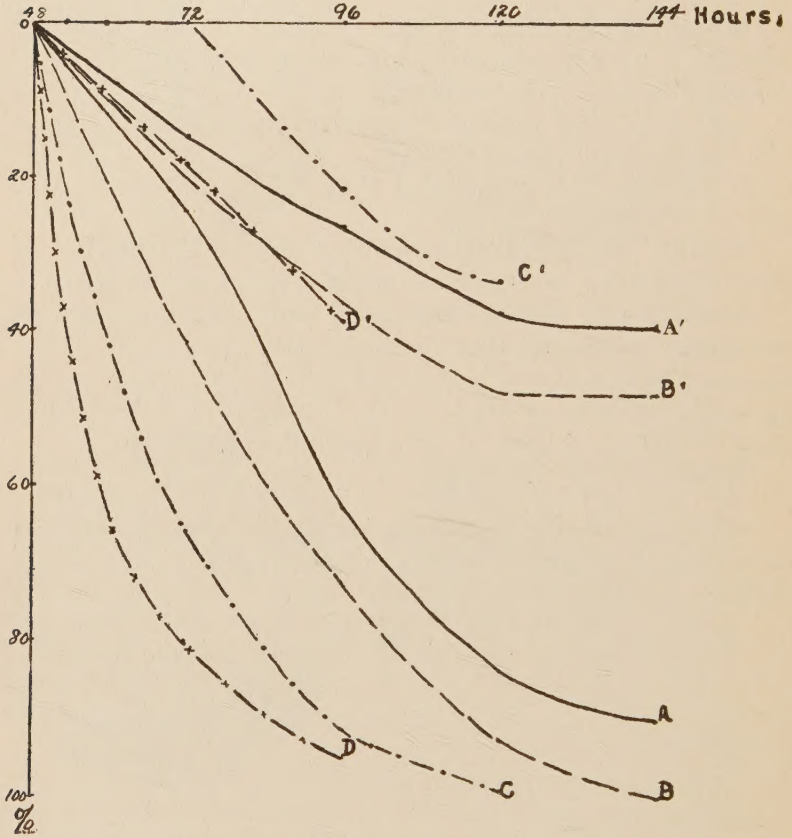
Philpott (17) 1929, ascertained,

"In the study of the effect of tetanus and botulinus toxins, of ricin and of snake venoms upon protozoa, only the venoms have definitely given positive results."

I

EFFECT OF COMMERCIAL DIPHTHERIA TOXIN ON GROWTH OF FIBROBLASTS

(Decrease Recorded in Percentage)



—— A, 1–400 dilution
— —B, 1–300 "
—·—C, 1–200 "
—x—D, 1–100 "

Commercial Toxin P55 (with preservative)

A, B, C, D—the respective experiments.
A′, B′, C′, D′—corresponding controls.

Experiments:

I. The Effect of Commercial Diphtheria Toxin on Migrating Fibroblasts

See Graph I

Diphtheria Toxin P55, M. L. D. = 1/400 per cc, L+0.2, Trikresol 0.5 per cent.

A. Experiments (1–400 dilution, 1 M. L. D. per cc): At the end of 48 hours, 148 pieces of 7-day chick embryo heart showed living fibroblasts. These pieces were bathed with a 1–400 dilution of diphtheria toxin and 24 hours later (i. e. at the end of 72 hours), there were 112 pieces showing living fibroblasts, a decrease of 24.4 per cent. This is designated as "A". At the end of 96 hours, 55 pieces showed active fibroblasts, a decrease of 62.9 per cent. At the end of 120 hours, 24 pieces showed migrating fibroblasts, a decrease of 83.8 per cent. Finally at the end of 144 hours, 14 pieces remained which showed fibroblasts, which is a decrease of 90.6 per cent.

When we note that pieces show fibroblasts, we are designating the migrating cells with their pseudopodia-cytoplasmic projections or processes—which vary in size and shape. (See Plate I, a, b.)

In direct contrast, in recording no living fibroblasts, we designate pieces showing no migrating cells. In some cases as the result of cell death, the fibroblasts become spindle shaped, with many vacuoles; while in others they round up. (See Plates II and III.)

With the 1–400 dilution of toxin, some pieces of heart tissue were observed to be pulsating at the end of 72 hours, i. e. 24 hours after they had been bathed with the toxin. A few were pulsating at the end of 96 hours, and one piece was observed pulsating at the end of 120 hours. However upon reviewing the literature it was noted that: Lake (7) 1916, in using embryonal heart tissue of rabbits, mice and human beings found that, "The older the heart tissue the smaller the number which pulsate." Burrows (2) 1912, states that pulsation depends upon the age of the embryo, and place from which you have taken the

piece of heart. The frequency of rhythm is greater in the auricle than in the ventricle. Therefore as pulsation of the heart was not the object of this study no further records were kept of this phenomenon. Nevertheless, it is interesting to note the mere fact that pulsation did occur after these pieces of heart tissue had been bathed with a 1–400 dilution of diphtheria toxin. It has been a common belief that death at the height of diphtheria was due to "heart failure". MacCallum (15) 1922, says:

"In very acute and intense infections or toxic processes, such as diphtheria and scarlet fever, there may be actual necrosis or hyaline degeneration of fibers here and there. . . . It is possible that these things, involving as they do the loss of many of the muscle fibers, should not weaken the heart. Such is its reserve power, however, that it generally continues to beat well enough in spite of them. They have been regarded by many authors(Stejskal and others) as the cause of the sudden collapse and death at the height of diphtheria, but Pässler and Romberg, MacCallum, and others have shown that deaths of this sort are due rather to the failure of the vaso motor control of the arteries (or possibly to the effect of Dale's capillary poisons), which allows the blood pressure to fall and the whole heart to suffer acutely from lack of nutrition."

Controls: At the end of the 48-hour period 116 pieces of chick embryo heart, showing fibroblasts, were used as controls. After being washed with Locke-Lewis solution and a 24-hour period being allowed to elapse, we find at this 72-hour reading, 99 pieces showed fibroblasts, a decrease of 14.7 per cent. This is designated as "A'". At the end of 96 hours, 85 pieces showed fibroblasts, a decrease of 26.8 per cent. At the end of 120 hours, 72 pieces showed fibroblasts, a decrease of 38.0 per cent. At the end of 144 hours, 70 pieces showed fibroblasts, a decrease of 39.7 per cent.

B. Experiments (1–300 dilution, 1.3 M. L. D. per cc): At the end of 48 hours, 109 pieces of chick embryo heart showed fibroblasts. These pieces were bathed with a 1–300 dilution of diphtheria toxin P55 and 24 hours later (i. e. at the end of 72 hours), 64 pieces were observed showing fibroblasts, a decrease of 41.3 per cent. This is designated as "B". At the end of 96 hours, 31 pieces showed fibroblasts, a decrease of 72.4 per cent.

At the end of 120 hours, 10 pieces showed fibroblasts, a decrease of 90.9 per cent. When the 144-hour reading was made 1 piece showed fibroblasts, a decrease of 99.1 per cent.

Controls: At the end of the 48-hour period 75 pieces, all of which showed fibroblasts, were used as controls. These were bathed with Locke-Lewis solution, and after allowing a 24-hour period to elapse, we observed at the end of 72 hours that there were 60 pieces which showed fibroblasts, a decrease of 20 per cent. This is designated as "B′". At the end of 96 hours, 48 pieces were observed showing fibroblasts, a decrease of 36 per cent. At the end of 120 hours, 39 pieces were observed showing fibroblasts, a decrease of 48 per cent. At the end of 144 hours it was observed that there was no loss in fibroblasts, i. e. that there were still 39 pieces showing fibroblasts.

As has been observed from these two sets of experiments, the most significant decrease in growth occurs 24 hours to 48 hours after the toxin dilutions have been applied, i. e. at the end of the 72-hour and 120-hour readings. Therefore future experiments were not carried on for the 144-hour period, but include the 48-, 72- and 96-hour readings.

C. Experiments (1–200 dilution, 2 M. L. D. per cc): After a 48-hour growth period, 119 pieces of chick embryo heart, all of which showed fibroblasts, were bathed with a 1–200 dilution of diphtheria toxin P55. After allowing a 24-hour period to elapse, we find at this 72-hour reading, 40 pieces showing fibroblasts, a decrease of 66.4 per cent. This is designated as "C". At the end of 96 hours, 10 pieces showed fibroblasts, a decrease of 91.6 per cent. At the end of 120 hours, 2 pieces showed fibroblasts, a decrease of 98.4 per cent.

Controls: At the end of a 48-hour growth period, 50 pieces, all of which showed fibroblasts, were used as controls. After being bathed with Locke-Lewis solution and a 24-hour period being allowed to elapse, we find at this 72-hour reading, 50 pieces showed fibroblasts, a decrease of 0 per cent. This is designated as "C′". At the end of 96 hours, 39 pieces showed fibroblasts, a decrease of 22 per cent. At the end of 120 hours, 33 pieces showed fibroblasts, a decrease of 34 per cent.

D. Experiments (1–100 dilution, 4 M. L. D. per cc): After a

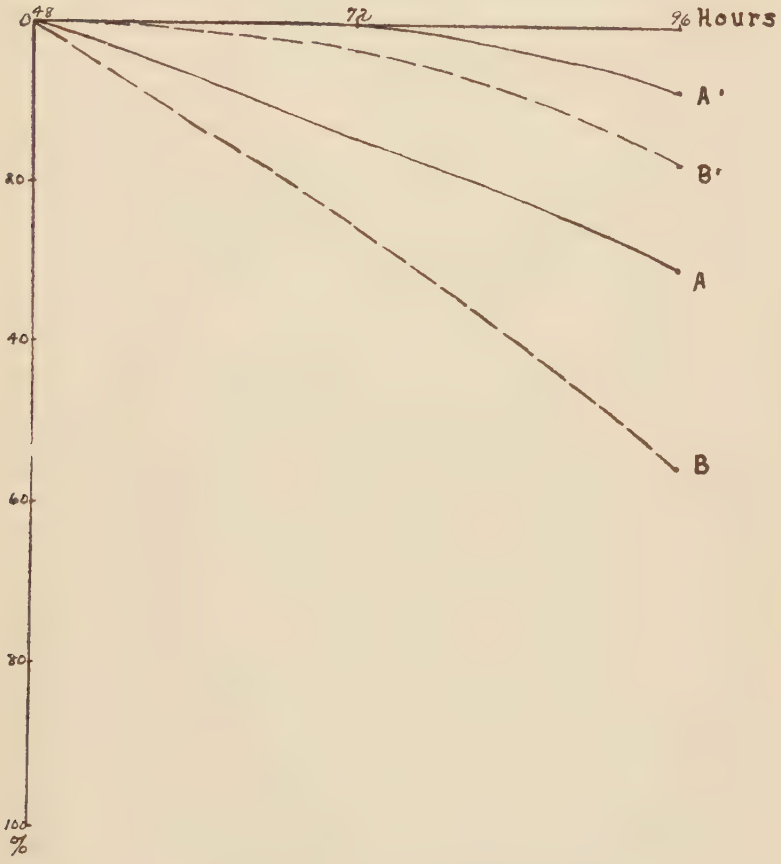
growth period of 48 hours, 136 pieces of chick embryo heart showing fibroblasts, were bathed with a 1–100 dilution of diphtheria toxin P55. After allowing a 24-hour period to elapse, we find at this 72-hour reading, 27 pieces showed fibro-blasts, a decrease of 80.2 per cent. This is designated as "D". At the end of 96 hours, 7 pieces showed fibroblasts, a decrease of 94.9 per cent.

Controls: At the end of the 48-hour period, 75 pieces, all of which showed migrating fibroblasts, were used as controls. After being bathed with Locke-Lewis solution and a 24-hour period being allowed to elapse, we find at this 72-hour reading, 61 pieces showed fibroblasts, a decrease of 18.7 per cent. This is designated as "D′". At the end of 96 hours, 46 pieces showed fibroblasts, a decrease of 38.7 per cent.

Thus it is seen that toxin P55 when used in a 1–100 dilution "D", after a contact of 24 hours caused a decrease of 80.2 per cent in growth, and a decrease of 94.9 per cent for a 48-hour contact. A 1–200 dilution "C", after a contact of 24 hours caused a growth decrease of 66.4 per cent, a decrease of 91.6 per cent for a 48-hour contact, and a decrease of 98.4 per cent for a 72-hour contact. A 1–300 dilution "B", after a contact of 24 hours caused a growth decrease of 41.3 per cent, a decrease of 72.4 per cent for a 48-hour contact, a decrease of 90.9 per cent for a 72-hour contact, and a decrease of 99.1 per cent for a 96-hour contact. A 1–400 dilution "A", after a contact of 24 hours caused a growth decrease of 24.4 per cent, a decrease of 62.9 per cent for a 48-hour contact, a decrease of 83.8 per cent for a 72-hour contact, and a decrease of 90.6 per cent for a 96-hour contact. As compared with the controls, in which we have an average decrease in growth of 13.35 per cent, 30.87 per cent, 40.00 per cent, 43.85 per cent, for the same time limits.

II

Effect of Diphtheria Toxin Without Preservative on Growth of Fibroblasts



—— A, 1–600 dilution
— —B, 1–150 "

Toxin No. 298

A, B—respective experiments.
A′, B′—corresponding controls.

II. The Effect of Diphtheria Toxin without Preservative on Growth of Fibroblasts

See Graph II

Following the same technique that we used for our commercial toxin experiments, 48-hour growths of migrating fibroblasts from explants of 7-day chick embryo hearts, were bathed with varying dilutions of diphtheria toxin No. 298, M. L. D. = 1/600 per cc.

A. Experiments (1–600 dilution, 1 M. L. D. per cc): At the 48-hour reading 125 pieces showing fibroblasts, were bathed with a 1–600 dilution of diphtheria toxin No. 298 without preservative, and a reading was made 24 hours later. At the end of 72 hours, 107 pieces showing fibroblasts remained, a decrease of 14.4 per cent. This is designated as "A". The reading made 24 hours later (i. e. at the end of 96 hours), showed 93 pieces with fibroblasts, a decrease of 25.6 per cent. Numerous pulsations were noted both in the 72- and 96-hour readings.

Controls: At the end of the 48-hour period, 22 pieces showing fibroblasts were bathed with Locke-Lewis solution and a reading was made 24 hours later. At the end of 72 hours, there was no decrease in the number of pieces showing fibroblasts. The reading made 24 hours later (i. e. at the end of 96 hours) showed 20 pieces with fibroblasts, a decrease of 9.1 per cent. This is designated as "A'".

B. Experiments (1–150 dilution, 4 M. L. D. per cc): At the end of the 48-hour period, 106 pieces showing fibroblasts were bathed with a 1–150 dilution of diphtheria toxin No. 298 and a reading was made 24 hours later. At the end of 72 hours, 79 pieces which showed fibroblasts remained, a decrease of 25.5 per cent. This is designated as "B". The reading made 24 hours later (i. e. at the end of 96 hours), showed 47 pieces with fibroblastic growth, a decrease of 55.7 per cent.

Controls: At the end of the 48-hour period, 29 pieces showing fibroblasts, were bathed with Locke-Lewis solution and a reading was made 24 hours later. At the end of 72 hours, 28 pieces which showed fibroblasts remained, a decrease of 3.5 per cent. This is designated as "B'". The reading made 24 hours later

(i. e. at the end of 96 hours), showed 24 pieces with fibroblasts, a decrease of 17.3 per cent.

III. Comparison of the Effect of Diphtheria Toxin With Preservative, With Diphtheria Toxin Without Preservative Upon Migrating Fibroblasts

See Graph III

Referring to the results of forgoing experiments, we find:

In experiments with commercial toxin (preserved with 0.5 per cent trikresol), 1–400 dilution (1 M. L. D. per cc) experiments, at the 72- and 96-hour readings, explants show a decrease in migrating fibroblasts of 24.4 per cent and 62.9 per cent (W), respectively; as compared with the corresponding controls, where we have decreases of 14.7 per cent and 26.8 per cent (W′), respectively.

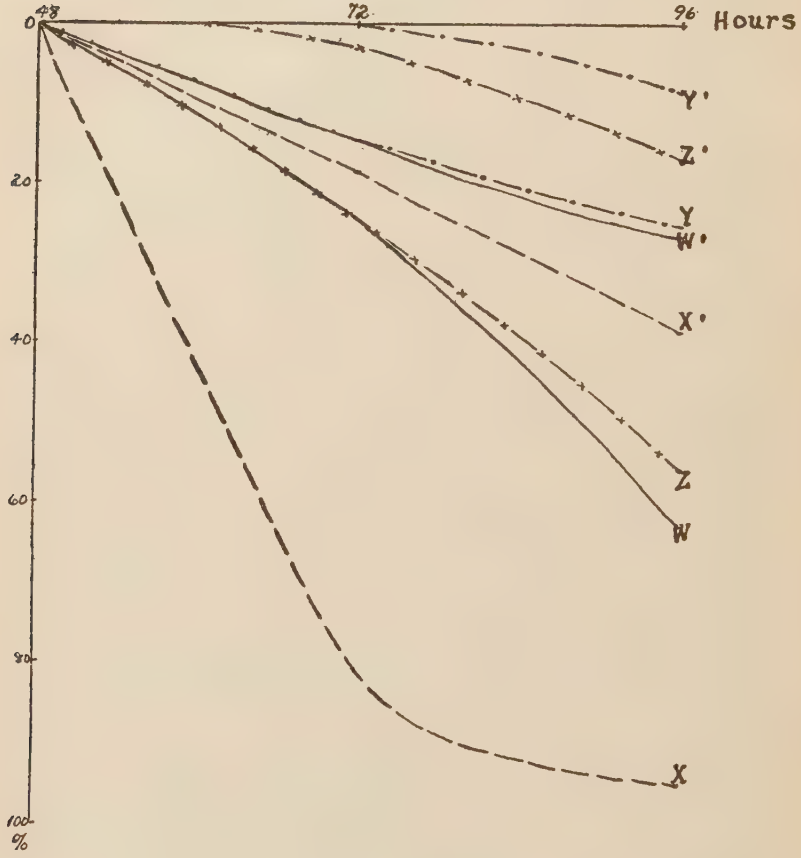
In corresponding experiments, in which a 1–100 dilution (4 M. L. D. per cc) was used, at the 72- and 96-hour readings, explants show a decrease in migrating fibroblasts of 80.2 per cent and 94.9 per cent (X), respectively; as compared with the corresponding controls, where we have decreases of 18.7 per cent and 38.7 per cent (X′), respectively.

In experiments with toxin No. 298 (without preservative), in which a 1–600 dilution (1 M. L. D. per cc) was used, at the 72- and 96-hour readings, explants show a decrease in migration of fibroblasts of 14.4 per cent and 25.6 per cent (Y), respectively; as compared with the corresponding controls, where we have a decrease in fibroblasts only at the 96-hour reading to the extent of 9.1 per cent (Y′).

In corresponding experiments in which a 1–150 dilution (4 M. L. D. per cc) was used, at the 72- and 96-hour readings, explants show a decrease in migration of fibroblasts of 25.5 per cent and 55.7 per cent (Z), respectively; compared with the corresponding controls, where we have decreases of 3.5 per cent and 17.3 per cent (Z′) respectively.

III

Comparison of Effects of Diphtheria Toxin, With and Without Preservative on Growth of Fibroblasts, Based on Growth of Controls and Treated Explants



——— W, 1 M. L. D. toxin with preservative. (1–400 dilution)
— — —X, 4 M. L. D. toxin with preservative. (1–100 ")
—·—·—Y, 1 M. L. D. toxin without preservative. (1–600 ")
—x—x—Z, 4 M. L. D. toxin without preservative. (1–100 ")
W, X, Y, Z—the respective experiments.
W′, X′, Y′, Z′—corresponding controls.

IV. The Effect of Commercial Diphtheria Toxin Heated to 60°C. for 30 minutes, on Fibroblasts

See Graph IV

Banzhaf (1) 1928, states:

"Heating *B. diphtheriae* toxin to 50°C. for 12 hours destroys about half of the acutely toxic fraction. . . . "

Levaditi (11) 1928, states:

"The heating of toxin to 100°(C.) for 10 or 15 minutes takes away its toxicity for the guinea pig, but it does not render it completely inoffensive for the fibroblasts cultivated outside the organism."

To have complete control on our Toxin P55 experiments, we have heated this toxin to 60°C. for one-half hour, thereby destroying the exotoxin (16). A dilution of 1–100 was used for experimental tests, following the same technique as was used in the previous toxin experiments.

Toxin P55 heated to 60°C. for 30 minutes (1–100 dilution):

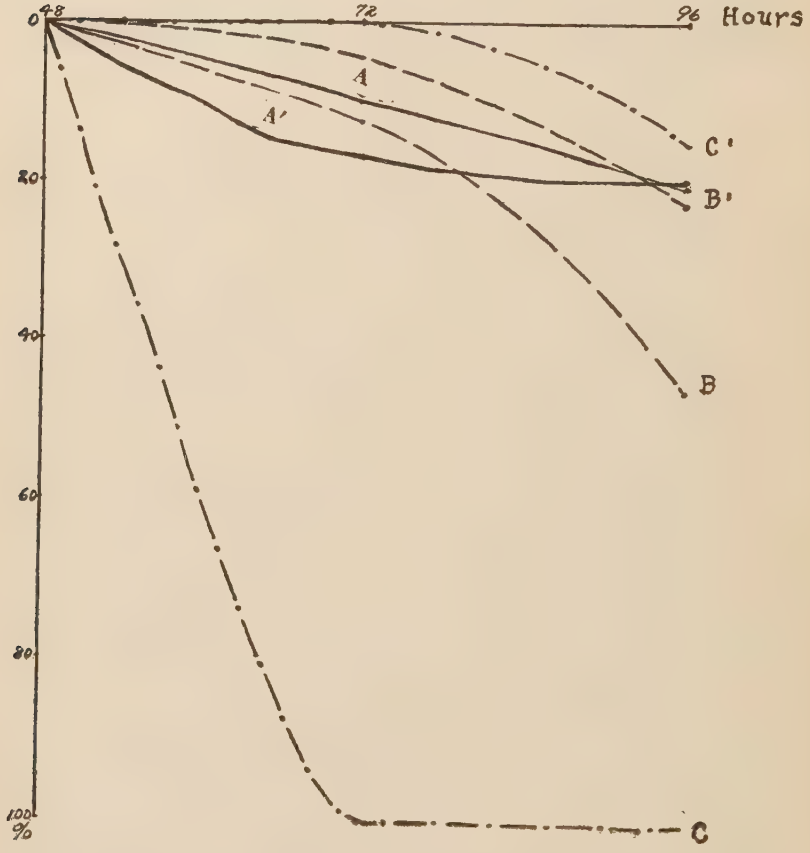
A. Experiments: At the 48-hour reading 121 pieces showing fibroblasts were bathed with a 1–100 dilution of Toxin P55 which had been heated previously to 60°C. for 30 minutes, and a reading was made 24 hours later. At the end of 72 hours, 109 pieces showing fibroblasts remained, a decrease of 10 per cent. This is designated as "A". The reading made 24 hours later (i. e. at the end of 96 hours), showed 96 pieces with migrating fibroblasts, a decrease of 20.7 per cent.

Controls: At the end of the 48-hour period, 30 pieces showing fibroblasts were bathed with Locke-Lewis solution, a reading was made 24 hours later. At the end of 72 hours, 25 pieces showing fibroblasts remained, a decrease of 16.7 per cent. This is designated as "A′". The reading made 24 hours later (i. e. at the end of 96 hours) showed 24 pieces with fibroblasts, a decrease of 20.0 per cent.

One must consider the fact that 30 pieces were used for controls, as compared with 121 pieces for experiments; and that there may be variations in migration of fibroblasts when pieces are taken

IV

EFFECT OF COMMERCIAL DIPHTHERIA TOXIN HEATED TO 60°C. FOR 30 MINUTES, ON GROWTH OF FIBROBLASTS



—— A, 1–100 dilution
— —B, 1–50 "
—.—C, undiluted

Commercial Diphtheria Toxin (P55) heated to 60°C. for 30 min.

A, B, C—the respective experiments.
A', B', C'—the corresponding controls.

from the same embryo, we are told by Ebeling (4) 1921, in reference to tissues grown in plasma—and for these experiments a number of embryos were used. Then too, chance plays a rôle, in that the pieces used for controls were not specially selected, for the last two or three slides that had been read were simply marked "controls", at the end of the 48-hour reading.

Toxin P55 heated to 60°C. for 30 minutes (1–50 dilution):

B. Experiments: At the 48-hour reading, 102 pieces showing fibroblasts were bathed with toxin P55, previously heated to 60°C. for 30 minutes and then diluted 1–50, and a reading was made 24 hours later. At the end of 72 hours, 89 pieces showing fibroblasts remained, a decrease of 12.8 per cent. This is designated as "B". The reading made 24 hours later (i. e. at the end of 96 hours), showed 55 pieces with fibroblasts, a decrease of 46.1 per cent.

Controls: At the end of the 48-hour reading, 23 pieces showing fibroblasts were bathed with Locke-Lewis solution, a reading being made 24 hours later. At the end of 72 hours, 22 pieces showing fibroblasts remained, a decrease of 4.4 per cent. This is designated as "B'". The reading made 24 hours later (i. e. at the end of 96 hours) showed 20 pieces with fibroblasts, a decrease of 13.1 per cent.

Toxin P55 heated to 60°C. for 30 minutes (undiluted):

C. Experiments: At the 48-hour reading 109 pieces showing fibroblasts were bathed with undiluted toxin P55, which had been subjected to 60°C. for 30 minutes. A reading was made 24 hours later. At the end of 72 hours, no pieces were observed which showed fibroblasts, a decrease of 100 per cent. This is designated as "C". At this time the cultures were discarded.

Controls: At the end of the 48-hour period, 33 pieces showing fibroblasts were bathed with Locke-Lewis solution and a reading was made 24 hours later. At the end of 72 hours, 33 pieces showing fibroblasts still remained. This is designated as "C'". The reading made 24 hours later (i. e. at the end of 96 hours), showed 28 pieces with fibroblasts, a decrease of 15.2 per cent.

V. Effect of 0.5 per cent Trikresol on Growth of Fibroblasts

See Graph V

Due to the fact that commercial toxin P55 has a preservative 0.5 per cent trikresol, it was thought advisable to make a series of tests using this preservative, made to a 0.5 per cent solution with Locke-Lewis solution, this in turn being diluted 1–100 and 1–400.

A. Experiments (undiluted 0.5 per cent trikresol): At the 48-hour reading, 107 pieces showing fibroblasts were bathed with 0.5 per cent trikresol, a reading being made 24 hours later. At the end of 72 and 96 hours, no pieces remained which showed migration of fibroblasts, a decrease of 100 per cent. This is designated as "A".

Controls: At the 48-hour reading, 27 pieces showing fibroblasts were bathed with Locke-Lewis solution, a reading being made 24 hours later. At the end of 72 hours, 25 pieces showing fibroblasts remained, a decrease of 7.5 per cent. This is designated as "A'". The reading made 24 hours later (i. e. at the end of 96 hours), showed 24 pieces with fibroblasts, a decrease of 11.2 per cent.

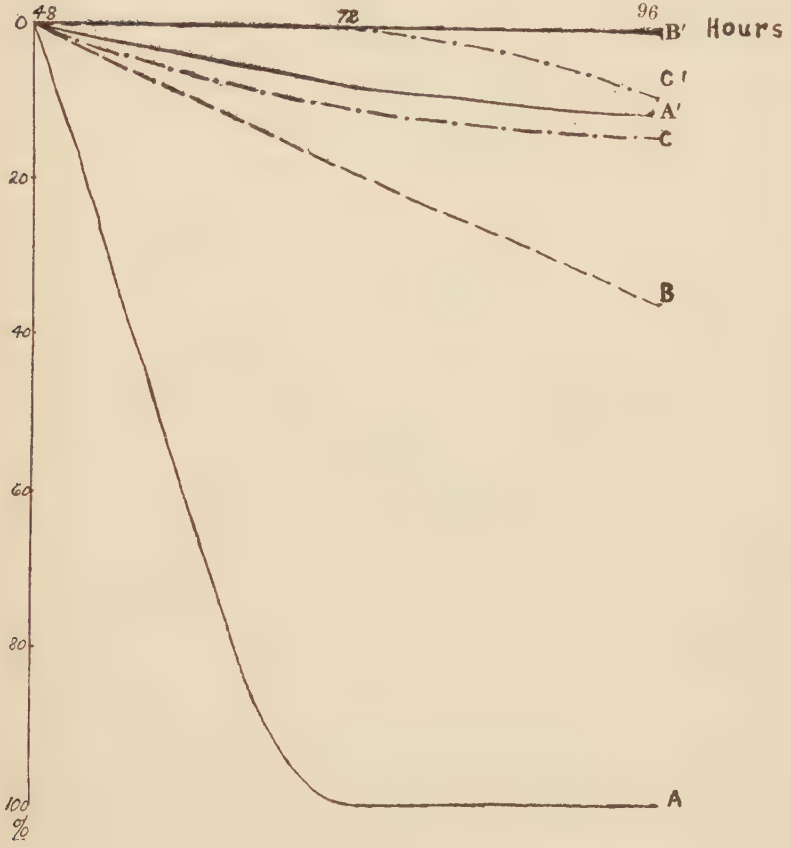
B. Experiments (0.5 per cent trikresol diluted 1–100): At the 48-hour reading, 162 pieces showing fibroblasts were bathed with a 1–100 dilution of 0.5 per cent trikresol, and a reading was made 24 hours later. At the end of 72 hours, 132 pieces showing living fibroblasts remained, a decrease of 18.6 per cent. This is designated as "B". The reading made 24 hours later (i. e. at the end of 96 hours) showed 104 pieces with fibroblasts, a decrease of 35.8 per cent.

Controls: At the end of the 48-hour period, 31 pieces showing fibroblasts were bathed with Locke-Lewis solution, readings being made 24 and 48 hours later. At the end of 72 and 96 hours, these 31 pieces still showed fibroblasts. This is designated as "B'".

C. Experiments (0.5 per cent Trikresol diluted 1–400): At the 48-hour reading, 114 pieces showing fibroblasts were bathed with a 1–400 dilution of 0.5 per cent trikresol, and a reading was made 24 hours later. At the end of 72 hours, 102 pieces showing

V

EFFECT OF 0.5% TRIKRESOL ON GROWTH OF FIBROBLASTS



——— A, undiluted
— — — B, 1–100 dilution
—·—·— C, 1–400 dilution
A, B, C—the respective experiments.
A′, B′, C′—corresponding controls.

living fibroblasts remained, a decrease of 10.6 per cent. This is designated as "C". The reading made 24 hours later (i. e. at the end of 96 hours) showed 97 pieces with migrating fibroblasts, a decrease of 15.0 per cent.

Controls: At the end of the 48-hour period, 24 pieces showing fibroblasts were bathed with Locke-Lewis solution, a reading being made 24 hours later. At the end of 72 hours, there was no decrease in fibroblasts. The reading made 24 hours later (i. e. at the end of 96 hours), showed 22 pieces with fibroblasts, a decrease of 8.4 per cent. This is designated as "C'".

VI. Effect of Toxoid Upon Growth of Fibroblasts

See Graph VI

The toxoid used in these experiments was made by treating toxin with 0.3 per cent formalin and incubating at 42°C. This formalin-treated-toxin was then diluted 1:5 which really makes a solution containing 0.06 per cent formalin; with an antigenic value of 0.1cc, which when given once protects guinea pigs against 5 M. L. D. of standard toxin given 30 days later. It contains 1:10,000 mercurophen as preservative.

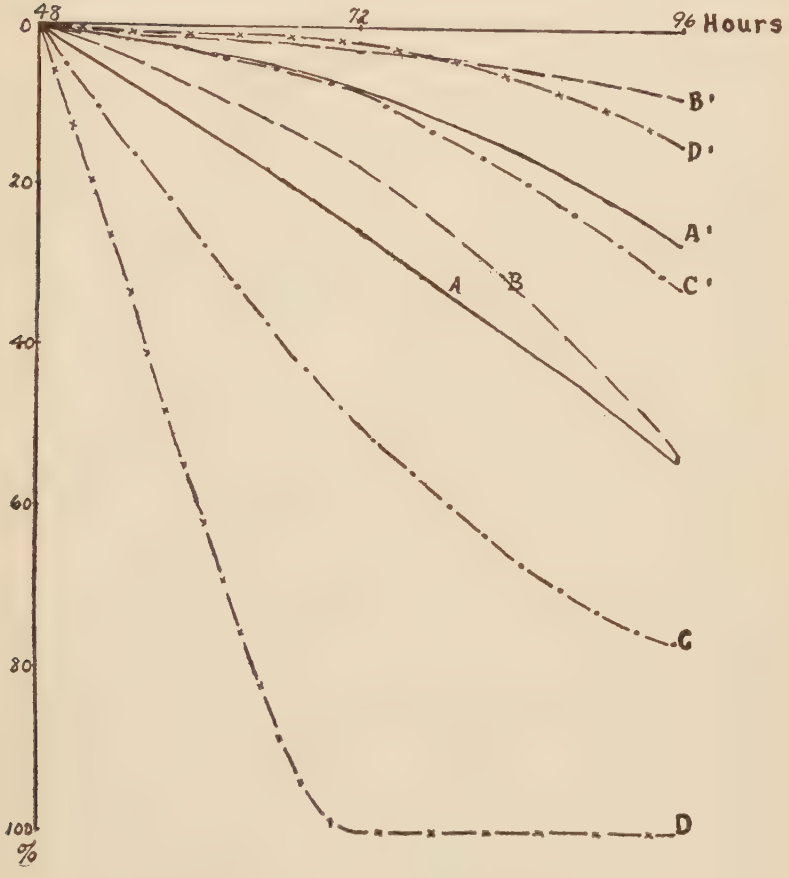
Toxoid: Following the same technique, 48-hour growth of migrating fibroblasts from explants of 7-day chick embryo hearts were bathed with varying dilutions of toxoid (26727–2 originally 20342).

A. Experiments (1–100 dilution of toxoid): At the 48-hour reading, 147 pieces showing fibroblasts were bathed with a 1–100 dilution of toxoid and a reading was made 24 hours later. At the end of 72 hours, 109 pieces showing fibroblasts remained, a decrease of 25.9 per cent. This is designated as "A". The reading made 24 hours later (i. e. at the end of 96 hours), showed 68 pieces with fibroblasts, a decrease of 53.8 per cent.

Controls: At the end of the 48-hour period, 52 pieces showing fibroblasts were bathed with Locke-Lewis solution and a reading was made 24 hours later. At the end of 72 hours, 48 pieces which showed fibroblasts remained, a decrease of 7.7 per cent. This is designated as "A'". The reading made 24 hours later

VI

EFFECT OF TOXOID ON GROWTH OF FIBROBLASTS



—— A, 1–100 dilution
— —B, 1–50 "
—·—C, 1–10 "
—x—D, undiluted

Toxoid (26727–2)

A, B, C, D—the respective experiments.
A′, B′, C′, D′—corresponding controls.

(i. e. at the end of 96 hours), showed 38 pieces with fibroblasts, a decrease of 27 per cent.

B. Experiments (1–50 dilution of toxoid): At the 48-hour reading, 110 pieces showing fibroblasts were bathed with a 1–50 dilution of toxoid and a reading was made 24 hours later. At the end of 72 hours, 91 pieces showing fibroblasts remained, a decrease of 17.3 per cent. This is designated as "B". The reading made 24 hours later (i. e. at the end of 96 hours), showed 71 pieces with migrating fibroblasts, a decrease of 53.8 per cent.

Controls: At the end of the 48-hour reading, 35 pieces showing fibroblasts were bathed with Locke-Lewis solution, and a reading was made 24 hours later. At the end of 72 hours, 34 pieces showing living fibroblasts remained, a decrease of 2.9 per cent. This is designated as "B′". The reading made 24 hours later (i. e. at the end of 96 hours) showed 32 pieces with fibroblasts, a decrease of 8.6 per cent.

C. Experiments (1–10 dilution of toxoid): At the 48-hour reading, 157 pieces showing fibroblasts were bathed with a 1–10 dilution of toxoid and a reading was made 24 hours later. At the end of 72 hours, 79 pieces which showed fibroblasts remained, a decrease of 49.7 per cent. This is designated as "C". The reading made 24 hours later (i. e. at the end of 96 hours), showed 37 pieces with fibroblasts, a decrease of 76.5 per cent.

Controls: At the end of the 48-hour period, 53 pieces showing fibroblasts were bathed with Locke-Lewis solution and a reading was made 24 hours later. At the end of 72 hours, 49 pieces which showed fibroblasts remained, a decrease of 7.6 per cent. This is designated as "C′". The reading made 24 hours later (i. e. at the end of 96 hours), showed 36 pieces with migrating fibroblasts, a decrease of 32.1 per cent.

D. Experiments (undiluted toxoid): At the 48-hour reading, 117 pieces showing fibroblasts were bathed with an undiluted solution of toxoid and a reading was made 24 hours later. At the end of 72 hours, no pieces showing fibroblasts were observed, a decrease of 100 per cent. This was designated as "D". At this time the cultures were discarded.

Controls: At the end of the 48-hour period, 35 pieces showing fibroblasts were bathed with Locke-Lewis solution and a reading

was made 24 hours later. At the end of 72 hours, 34 pieces showing fibroblasts remained, a decrease of 2.0 per cent. This is designated as "D'". The reading made 24 hours later (i. e. at the end of 96 hours) showed 30 pieces with fibroblasts, a decrease of 14.3 per cent.

Thus it is seen that toxoid (26727–2) when used in undiluted strength ("D"), caused a 100 per cent decrease of fibroblasts, that is allowing an exposure of 24 hours. A 1–10 dilution ("C"), caused a 49.7 per cent decrease for a 24-hour contact and a 76.5 per cent decrease for a 48-hour contact. A 1–50 dilution ("B"), caused a decrease of 17.3 per cent for a 24-hour contact and a decrease of 53.8 per cent for a 48-hour contact. A 1–100 dilution ("A"), caused a decrease of 25.9 per cent for a 24-hour contact and a decrease of 53.8 per cent for a 48-hour contact. As compared with the controls in which we have an average decrease of 5.05 per cent and 25.1 per cent for the same time limits.

VII. Effect of 0.06 per cent Formalin-Locke-Lewis Solution on Growth of Fibroblasts

See Graph VII

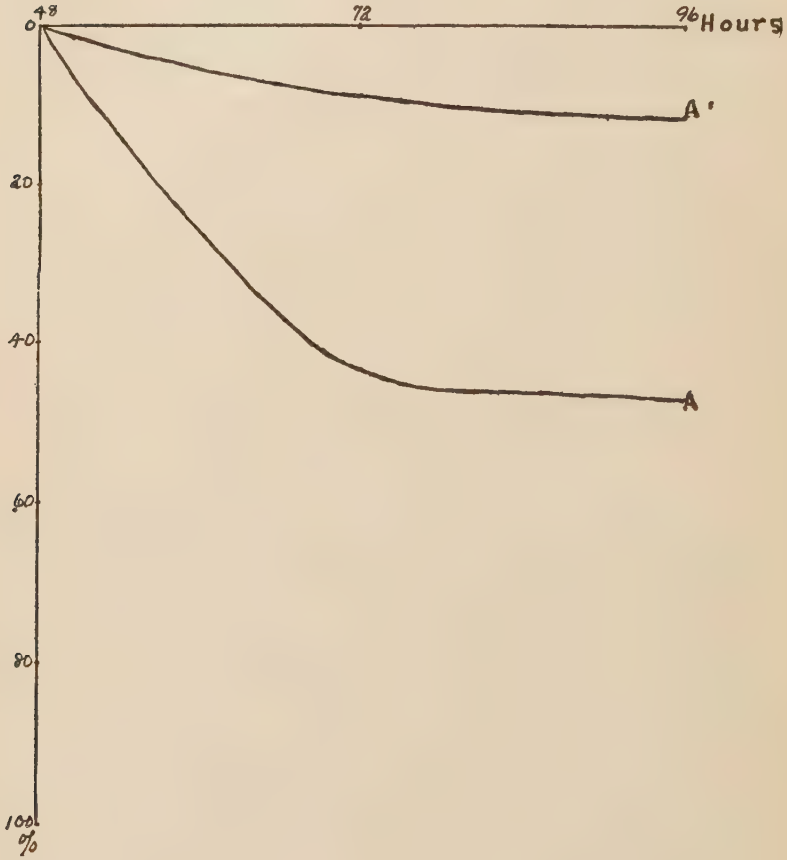
Due to the fact that the toxoid that was used originally had 0.3 per cent formalin, which when diluted 1–5 (the dilution received) contained 0.06 per cent formalin, the following work was undertaken. According to previous experiments no living fibroblasts were recorded when this undiluted toxoid was added to the explants of heart tissue. The question then arose, could this be due to 0.06 per cent formalin.

A Locke-Lewis solution with 0.06 per cent formalin was used for experimental purposes, and the same technique as was used in previous experiments was followed.

Experiments: At the 48-hour reading, 161 pieces showing fibroblasts, were bathed with this 0.06 per cent formalin-Locke-Lewis solution, and a reading was made 24 hours later. At the end of 72 hours, 92 pieces showing fibroblasts remained, a decrease of 42.9 per cent. This is designated as "A". The reading made 24 hours later (i. e. at the end of 96 hours), showed 83 pieces with fibroblasts, a decrease of 48.5 per cent.

VII

EFFECT OF 0.06% FORMALIN-LOCKE-LEWIS SOLUTION ON GROWTH OF FIBROBLASTS



A, 0.06% Formalin-Locke-Lewis Solution.
A', corresponding control.

Controls: At the end of the 48-hour period, 36 pieces showing fibroblasts were bathed with Locke-Lewis solution, a reading being made 24 hours later. At the end of 72 hours, 33 pieces which showed fibroblasts remained, a decrease of 8.4 per cent. This is designated as "A′". The reading made 24 hours later (i. e. at the end of 96 hours), showed 32 pieces with fibroblasts, a decrease of 11.2 per cent.

VIII. Effect of Commercial Diphtheria Toxin-Antitoxin Mixture

See Graph VIII

According to the technique used in the previous experiments' the following results were obtained:

Undiluted Commercial Diphtheria T-A Mixture (23136–1 originally 15969), 5 doses killed guinea pigs in 21 and 22 days, 1 human dose did not kill guinea pigs.

A. Experiments: At the 48-hour reading, 133 pieces showing fibroblasts were bathed with undiluted commercial diphtheria T-A Mixture, and a reading was made 24 hours later. At the end of 72 and 96 hours, no pieces remained which showed migration of fibroblasts, a decrease of 100 per cent. This is designated as "A".

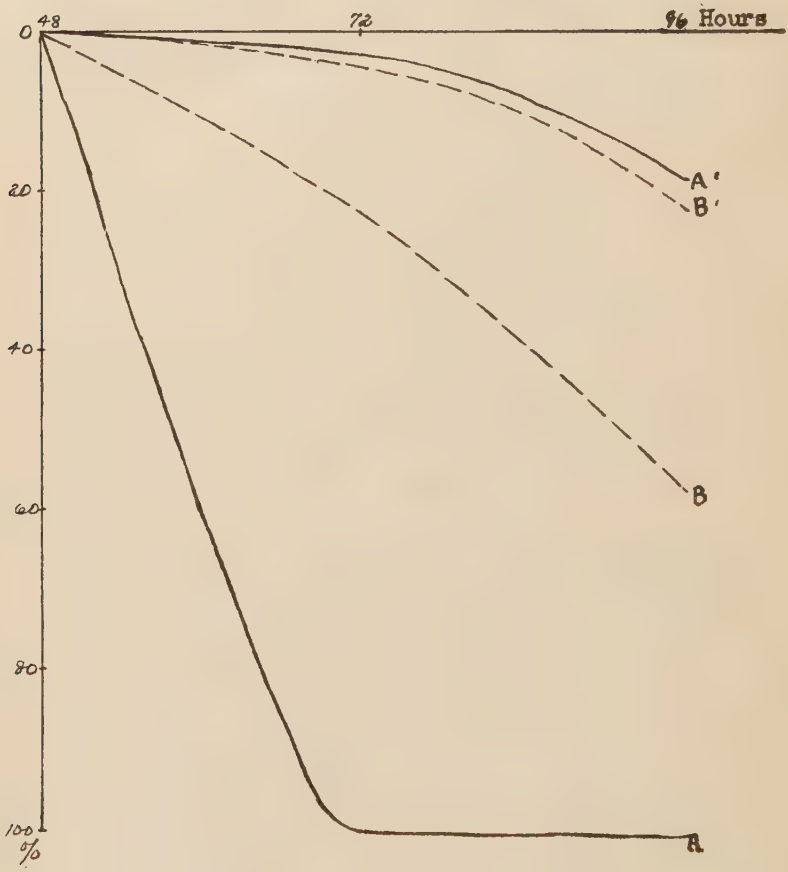
Controls: At the 48-hour reading, 34 pieces showing fibroblasts were bathed with Locke-Lewis solution, a reading being made 24 hours later. At the end of 72 hours, 33 pieces which showed fibroblasts remained, a decrease of 3 per cent. This is designated as "A′". The reading made 24 hours later (i. e. at the end of 96 hours) showed 31 pieces with fibroblasts, a decrease of 8.9 per cent.

Commercial Diphtheria T-A Mixture diluted 1–100:

B. Experiments: At the 48-hour reading, 108 pieces showing fibroblasts were bathed with commercial T-A mixture diluted 1–100, and a reading was made 24 hours later. At the end of 72 hours, 84 pieces showing fibroblasts remained, a decrease of 22.3 per cent. This is designated as "B". The reading made 24 hours later (i. e. at the end of 96 hours), showed 46 pieces with fibroblasts, a decrease of 57.5 per cent.

VIII

Effect of Commercial Diphtheria Toxin-Antitoxin Mixture, on Growth of Fibroblasts



—— A, undiluted toxin-antitoxin (23136–1).
— —B, 1–100 dilution.

Controls: At the end of the 48-hour reading, 24 pieces showing fibroblasts were bathed with Locke-Lewis solution, a reading being made 24 hours later. At the end of 72 hours, 23 pieces showing fibroblasts remained, a decrease of 4.1 per cent. This is designated as "B′". The reading made 24 hours later (i. e. at the end of 96 hours), showed 21 pieces with fibroblasts, a decrease of 12.5 per cent.

Summary

The experiments, on the effect of diphtheria toxin, toxoid and T-A Mixture upon migrating fibroblasts, according to the method described, were carried out as follows:

1. Commercial diphtheria toxin (with preservative) was used:
   (a) In 1–400 dilution (1 M. L. D. per cc) upon 148 explants showing migrating fibroblasts; at the same time 116 pieces were used as controls.
   (b) In 1–300 dilution upon 109 explants showing living fibroblasts, with 75 pieces as controls.
   (c) In 1–200 dilution upon 119 explants, with 50 pieces as controls.
   (d) In 1–100 dilution (4 M. L. D. per cc) upon 136 explants, with 75 pieces as controls.
2. Diphtheria toxin without preservative was used:
   (a) In 1–600 dilution (1 M. L. D. per cc) upon 125 explants showing living fibroblasts, with 22 explants as controls.
   (b) In 1–150 dilution (4 M. L. D. per cc) upon 106 explants, with 29 pieces as controls.
3. Commercial diphtheria toxin heated to 60°C. for 30 minutes, was used:
   (a) In 1–100 dilution upon 121 explants, with 30 pieces as controls.
   (b) In 1–50 dilution upon 102 explants, with 23 pieces as controls.
   (c) Undiluted upon 109 explants, with 33 pieces as controls.
4. The preservative, 0.5 per cent trikresol employed in commercial diphtheria toxin was used:
   (a) Undiluted, upon 107 explants, with 27 pieces as controls.

(b) In 1–100 dilution, upon 162 explants, with 31 pieces as controls.
(c) In 1–400 dilution, upon 114 explants, with 24 pieces as controls.

5. Toxoid was employed:
   (a) In 1–100 dilution, upon 147 explants, with 52 pieces as controls.
   (b) In 1–50 dilution, upon 110 explants, with 35 pieces as controls.
   (c) In 1–10 dilution, upon 157 explants, with 53 pieces as controls.
   (d) Undiluted, upon 117 explants, with 35 pieces as controls.
6. Formalin in a 0.06 per cent solution was used upon 116 explants, with 36 pieces as controls.
7. Commercial diphtheria T-A Mixture was used:
   (a) Undiluted, upon 133 explants, with 34 pieces as controls.
   (b) In 1–100 dilution, upon 108 explants, with 24 pieces as controls.

Conclusions

Of the diphtheria products tested for their action upon migrating fibroblasts of explants of seven-day chick embryo hearts, the only ones which showed a markedly deleterious effect in dilutions above 1 to 100 were the two active toxins. These experiments indicate that there is a deleterious effect in varying dilutions. The experiments using trikresol in the concentration in which it is found as a preservative in the commercial toxin, and experiments with commercial diphtheria toxin heated to 60°C. for 30 minutes, show that very little of the harmful effect which was evident in experiments with commercial toxin might be due to preservative. Toxoid and commercial T-A mixture used in varying dilutions, showed a deleterious effect upon fibroblasts; higher concentrations—as compared with the toxin —were necessary to show this result. Experiments with formalin in the concentration used in the preparation of toxoid, showed that this solution was not wholly responsible for the destruction of fibroblasts in experiments with undiluted toxoid. The fact

that, heating the toxin to 60°C. for 30 minutes or converting it into toxoid by the action of formalin annuls the toxic action almost completely, would seem to indicate that the agent which exerts the deleterious action on cultures is the thermolabile or true toxin. The fact that toxin No. 298, which was more active in tests on guinea pigs than toxin P55, proved less injurious to cultures of heart tissue is at present unexplained. Whether the difference is due to the presence of trikresol, bouillon, difference in pH or to some undetermined factor remains unsolved. The fact that there were considerable variations in the controls, suggests that the technique as employed, does not yield results that are as trustworthy as might be desired. In order to state whether this technique could serve in the standardization of toxins, a much larger series of experiments would have to be conducted on toxins with and without preservative.

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